



Early sex identification by leaflet distance in plantlets of *Cycas revoluta*

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Abstract In dioecious plants, the process of sex determination usually occurs during the reproductive stage. However, it can be challenging to determine the sex of long-lived plants with long generation times. This is true for *Cycas revoluta*, which produces toxic seeds in female plants, leading to a preference for male plants in public green spaces. In this study we aim to identify a morphological trait that can be used to distinguish between the sexes in *C. revoluta*

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at a very early stage of plant growth. We sampled *C. revoluta* seedlings/plantlets at three different early growth stages and identified the sex of individuals by PCR amplification of a male-specific molecular marker. On the same plants, we measured morphological traits, including the distance between leaflets on the leaf rachis, perimeter, area, number of indents, and leaf complexity, and evaluated their correlation with the sex of the individuals. Among all measured traits, the medium distance between leaflets on the leaf rachis was found to be a sexually dimorphic morphological trait in *Cycas* plantlets in all three growth stages. In particular, the distance between leaflets on the leaf rachis in the upper part of the leaf in the first stage revealed no overlap between the sexes. Whenever available, morphological traits for sex differentiation in greenhouses and plant nurseries can represent a rapid and economic tool when coupled with automatized image recognition systems.

Keywords Cycads · Plant sex determination · Plant sexual dimorphism · Secondary sexual characters · Sex-linked molecular marker

Introduction

The difference in the prevalence of hermaphroditism and monoecy, where a single organism expresses both sexes, versus dioecy, where an individual is either male or female, is one of the most important

factors distinguishing plants and animals in terms of sexual evolution (Mank 2022). Whereas most animals and the presumed ancestor are dioecious, this condition is derived and relatively rare in most land plants (Käfer et al. 2017; Renner and Müller 2021; Renner and Ricklefs 1995). While dioecy is rare in plants, it is nevertheless an evolutionary outcome of (basal) hermaphroditism in some lineages (Renner 2014). In dioecious plants, sex determination can be influenced by physiological or genetic factors. In *Cucumis* species, sexual expression can be affected by the environment and hormones, particularly by ethylene. This functions as a feminizing agent, and the ACS gene controls ethylene production, leading to the development of male and female reproductive organs (Papadopoulou et al. 2005). Sexually antagonistic conflicts in the initial stages of the evolution of dioecy are thought to be important in selecting for the evolution of sex-specific expression of genes and, in the long term, in suppressing recombination in the genome region carrying these genetic factors and leading to the evolution of sexual chromosomes (Charlesworth 2018). The origin of separate sexes from a hermaphrodite ancestor is commonly associated with the evolution of sexual dimorphism, and this has occurred to varying degrees in many dioecious plants (Corns 1929; Dawson and Geber 1999; Lloyd and Webb 1977). Sexual dimorphism refers to differences between the sexes in primary and secondary sex characters. The former refers directly to male (androecium) and female (gynoecium) sex organs, and the latter refers to differences between the sexes in structures other than the sex organs themselves, including any aspect of morphology or physiology (Barrett and Hough 2012). There are plenty of examples of sexual dimorphism in reproductive traits in dioecious species (Delph 1999; Eckhart 1999; Lloyd and Webb 1977). Sex-specific differences include flowering phenology and periodicity (Thomas and LaFrankie 1993), flower size (Delph et al. 1996), number of flowers per plant (Delph et al. 2005), flower longevity (Primack 1985), flower scent (Ashman 2009), flower defence against herbivory (Cornelissen and Stiling 2005) and various inflorescence traits including total flower number (Barrett and Hough 2012), daily floral display size (Yakimowski et al. 2011), and inflorescence architecture (e.g. Rourke 1989). In contrast, differences in secondary (vegetative) sex characters are less common and evident. This is partly because

plant sexual dimorphism is usually less obvious than in most animals.

At the seed or seedling stage, there are few reports of differences in sexual secondary characters. In *Rumex nivalis*, male seeds are heavier and germinate earlier than female seeds, but overall germination levels do not differ between the sexes (Stehlik and Barrett 2005). Male seeds are also heavier than female seeds in *Spinacia oleracea* (Freeman et al. 1994). On the other hand, several reports of size, morphology (leaf form, stem features, etc.), growth rate, and physiological differences between the sexes during the vegetative phase of growth have been reported (Dawson and Geber 1999; Lloyd and Webb 1977). However, for most dioecious plant species, sex differences only become evident in reproductive traits. Consequently, and in contrast to many animal groups, the sex of an individual in plants cannot usually be determined before the beginning of its reproductive stage (Garcia and Antor 1995), a major complication for early sex identification in long-lived plants with a long generation time. These become particularly relevant for those cultivated plants that require sex information well before the development of the reproductive organs. For instance, females of *Ginkgo biloba*, a dioecious tree that typically does not reach sexual maturity for 20 to 30 years (van Beek et al. 1998), produce seeds that emit a noxious, foul odour on falling to the ground (Wada and Haga 1997). These features caused city governments to remove and ban females from being planted (Echenard et al. 2008) and plant growers to have a strong interest in determining the sex of plants at an early stage of development. A similar case is that of Cycads, the most primitive extant seed-producing plants. These 'living fossils' are known to have existed at least since the Jurassic (Zhou and Zheng 2003) and, with their fascinating forms, Cycads are popular in landscape gardening. Among others, *Cycas revoluta* is used worldwide as an ornamental plant. *C. revoluta* has a distinctive trunk-like structure, resembling a palm tree and its foliage contains a cluster of long pinnate leaves that give it an exotic, tropical appearance. Although *C. revoluta* is an attractive ornamental plant, its seeds contain several toxic glycosides, as cycasin and neocycasin, which belong to the family of cyanogenic glycosides that pose a serious risk of

poisoning through accidental ingestion by kids and pets (Nishida et al. 1956). A study highlighted the clinical cases of *Cycas* seed poisoning in Taiwan and its cyanogenic potential (Chang et al. 2004). Thus, it is desirable to introduce only *C. revoluta* male plants in public green areas and to have tools for their early sex identification.

Traditionally, sex identification of *C. revoluta* is done by visual inspection of the reproductive structures of mature adult plants, i.e., after the formation of male and female cones. Cycads plants only reach sexual maturity after ca. 15–20 years (Segalla et al. 2021) and this can potentially result in significant costs for the cultivation of female plants, which should subsequently be discarded. Therefore, identifying the sex of *C. revoluta* plants at an early stage of plant development could be valuable for breeding and planning of plant production.

Once sex-linked genes/markers are known, plants, even at the seedling stage, can be genotyped for their sexes (Heikrujam et al. 2015). However, the availability of morphological tools would be preferable over molecular tools for their efficient application in nurseries and greenhouses (Inoti et al. 2015). Attempts have been made to identify dimorphic secondary sexual characters in Cycads, particularly in leaf morphology, but significant differences between sexes have never been reported at the juvenile stage (Newell 1985; Niklas and Marler 2008; Ornuduff 1996). More recently, Kaviani et al. (2014) compared a set of morphological traits between male and female two-year-old off-shoots of adult *C. revoluta* plants whose sex was previously determined (i.e. in plants that have already reached sexual maturity). Among others, they found the distance between leaflets 29 and 30 on the leaf rachis to be significantly different between sexes in male and female off-shoots. In the present work, we examined whether this trait and other related leaf traits such as area, perimeter, number of indents, and leaf complexity were different at the juvenile stage (from seedlings to plantlets) in *Cycas revoluta* plants whose sex was previously determined with a male sex-specific PCR marker (Liu et al. 2022). We aimed to find a vegetative morphological trait that could be used in greenhouses and plant nurseries as a rapid tool for easy identification of sex in *C. revoluta* plants at the earliest stage of development.

Materials and methods

Plant material

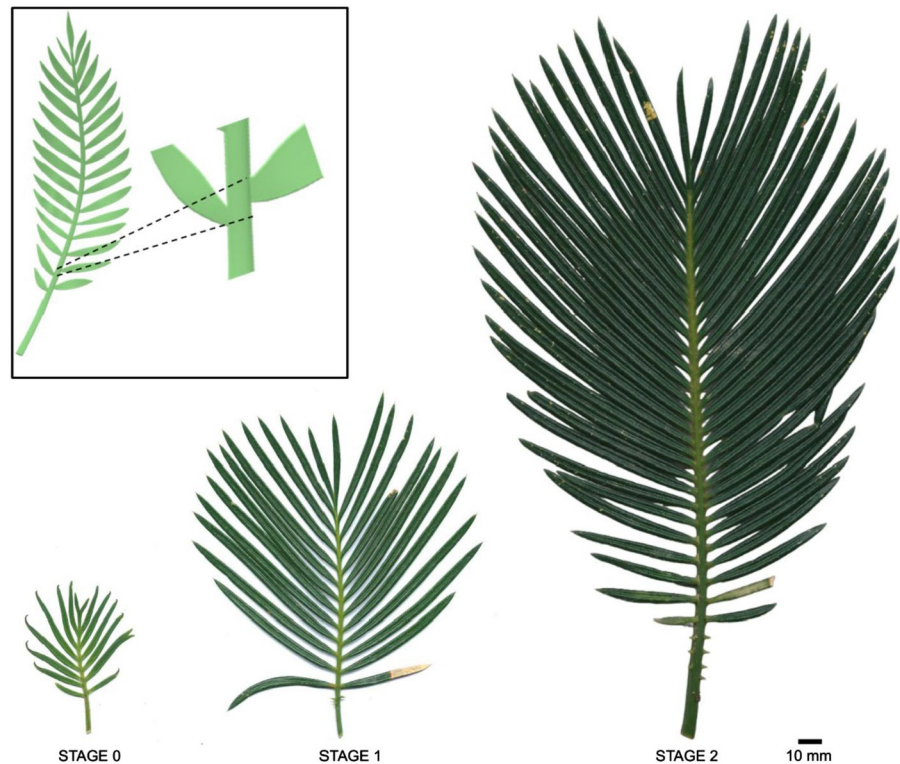
We sampled *C. revoluta* plantlets in March 2022 at the greenhouse Pianta Faro (Giarre, Sicily, Italy). Seedlings of *C. revoluta* were randomly sampled from the nursery greenhouse. Specifically, thirty individuals for three different growth stages were sampled from a pool of approximately 1000 individuals. We defined individuals belonging to stage 0 as those with leaf lengths varying between 20 and 50 mm, individuals belonging to stage 1 as those with leaf lengths varying between 50 and 100 mm, and individuals belonging to stage 2 as those with leaf lengths varying between 100 and 170 mm. These three stages roughly corresponded to seedlings of approx. 12 months (stage 0), 24 months (stage 1), and 36 months (stage 2) after seed germination, respectively (Fig. 1 and Fig. S1). We collected the oldest leaf from each individual (the single true leaf at stage 0, i.e. not the cotyledon).

In April 2024, we sampled an additional batch of 40 plants (all belonging to stage 0) at the greenhouse La Floricola (Ispica, Sicily, Italy).

Sex identification by molecular analysis

DNA from all individuals was extracted using a cetyltrimethylammonium bromide extraction protocol (Doyle and Doyle 1990) and quality and quantity were evaluated with the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, US). To determine the sex of individuals, a male-specific MADS-Y/CYCAS_034085 region was amplified by PCR using a sex-specific primer pair reported in Liu et al. (2022). With this primer pair, we expected to amplify a 720 bp fragment only in male individuals. We also amplified the autosome CYCAS_010388 region as a control both in males and females (Liu et al. 2022). As a preliminary control for PCR primers and amplification, we first examined 30 DNA samples from adult *C. revoluta* plants whose sex was already known by visual observation of their reproductive traits. All PCR reactions (25 µl final volume), with 10 ng of DNA as a template, 50 nM of each primer, 1×Taq polymerase buffer, 1.5 mM MgCl₂, 200 µM dNTP, and 0.5 unit Taq polymerase (Life Technologies, US), were conducted in an Applied Biosystems 2720

Fig. 1 Growth stages of *Cycas revoluta* plantlets. Stage 0 corresponds to plantlets of 12 months; stage 1 corresponds to plantlets of 24 months and stage 2 corresponds to plantlets of 36 months after seed germination. The drawing in the box shows the distance between leaflets on the leaf rachis (Inter Rachis Distance, IRD)



thermal cycler (Thermo Fisher, US) as follows: 3 min at 94° followed by 35 cycles of 30 s at 94°, 45 s at 54°, 50 s at 72° and 5 min at 72° for final extension. PCR products were then examined by electrophoresis in 1.5% agarose gel with a DNA molecular size marker.

Morphometric measurements

To measure the morphological traits, collected leaves were pressed and scanned (with an Epson printer scanner) with a 600-dpi resolution on a graph paper. This latter was used as a scale for software ImageJ (Schneider et al. 2012) to measure the distance between leaflets on the leaf rachis for each leaflet pair (i.e. Inter Rachis Distance, IRD).

To further characterize leaf morphology, leaf scans were also analyzed using Lamina software (Bylesjö et al. 2008), which generates estimates of leaf area, perimeter, and the number of indentations (indentations and leaf lobes). To estimate leaf complexity, we calculated perimeter^2 divided by area. It was impossible to measure these traits for stage 2 individuals

because their large leaves were leathery, preventing complete relaxation on the flat surface of the scanner.

Statistical analyses

The Shapiro–Wilk test was applied to all morphological traits to determine whether the values were normally distributed. If the p -value was less than $p=0.05$, there was sufficient evidence that the values were from a non-normally distributed population. In that case, we normalized the data by transforming them to a logarithmic scale (log). For normally distributed values, the Linear Regression Model (LM) and Linear Mixed Model (LMM) were applied using the lme4 package (Bates et al. 2015) in R (R Core Team 2023), where the fixed variable is the sex of the two groups (males and females), the dependent variable corresponds to the measure of the morphological traits, and the random factor for the LMM analysis corresponds to the individuals. The Akaike Information Criterion (AIC) was used to choose the model with higher relative quality (package stats in R; Bertrand 1988). To test whether the morphological differences between the two sexes were significant,

we used emmeans version 1.4 (Lenth et al. 2019) to conduct pairwise t-tests for multiple comparisons. For non-normally distributed values we used the generalized linear mixed model (GLMER), using the GAMMA function as a family, the morphological traits being quantitative, continuous, and discrete values. In this case, to assess whether there was a significant difference between the two sexes, we conducted the Mann–Whitney-U test in R. All statistical analyses were conducted with R version 4.2.3.

Results

Sex identification by molecular analysis

A positive amplification of both male-specific MADS-Y/CYCAS_034085 region and autosome CYCAS_010388 was an indication of the male sex of the plantlets while the absence of the male-specific PRC product (and a positive amplification of the control target) was taken as an indication of the female sex (Fig. S2). Regarding the plantlets sampled at the greenhouse Pianta Faro, for stage 0 we were able to assess the sex of 12 males and 16 females, (two samples failed PCR amplification), for stage 1 we assessed the sex of 20 females and 10 males, and for stage 2 we assessed the sex of 16 males and 14 females. For the 40 plantlets sampled at La Floricola greenhouse, we assessed the sex of 18 females and 22 males.

Morphometric measurements

Once the sex was determined, in the first analysis, we compared the IRD between each pair of leaflets on the leaf rachis for all plants and stages sampled at the greenhouse Pianta Faro (Table S1). We found different values for the two sexes but always with overlapping values. We also assessed whether medium leaf IRD (as the medium of all IRD measurements of the leaf) correlated with the sex of the plant. The results show that there is a statistically significant difference between the two sexes but with an overlapping distribution of values (Fig. 2). Finally, we analysed separate portions of the leaf by dividing it into three equal sections, namely the proximal, the median, and the distal part of the leaf petiole.

Our analyses revealed a significant correlation between the sex and IRD for all three leaf sections at stage 0. However, only for the section corresponding to the distal part of the leaf petiole, male and female trait measurements did never overlap and were discontinuous ($t=6.8996$, $df=73.907$, $p\text{-value}=1.519\text{e-}09$) (Fig. 3a). Instead, for the sections corresponding to the median and proximal part of the leaf petiole, the correlation was significant ($t=4.8991$, $df=72.974$, $p\text{-value}=5.624\text{e-}06$; $t=4.8684$, $df=56.434$, $p\text{-value}=9.454\text{e-}06$, respectively) but trait distribution between sexes overlapped due the presence of some outliers (Fig. 3b and c).

At plant stages 1 and 2, statistical analyses for the same three sections found significant correlations between IRD and the sex of the individuals but

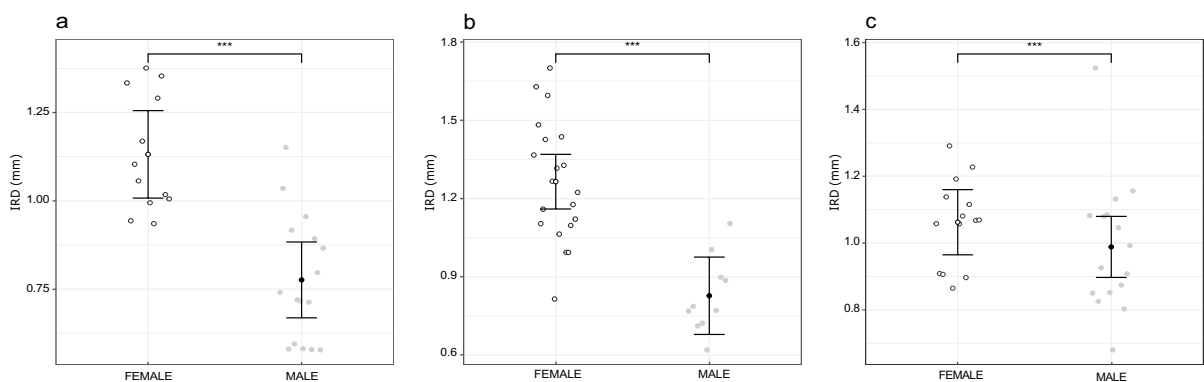


Fig. 2 Dimension of leaf IRD (as the medium of all measurements for each leaf with error bar, i.e. 95% confidence interval of the mean) in female and male plants at **a** stages 0 **b** stage 1

and **c** stage 2. White dots correspond to female individuals and grey dots represent male individuals. *** ($p\text{-value}<0.01$)

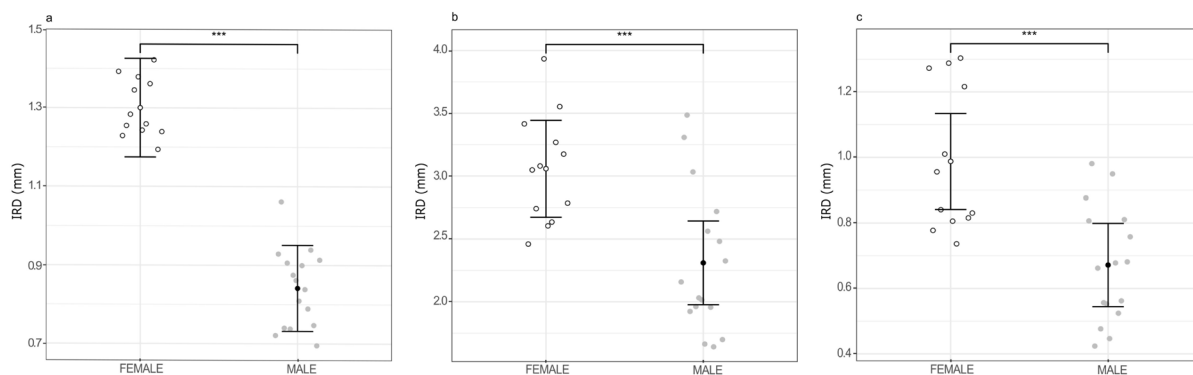


Fig. 3 Dimension of leaf IRD (as the medium of all measurements for each section with error bar, i.e. 95% confidence interval of the mean) in female and male plants at stage 0. White dots correspond to female individuals and grey dots

represent male individuals. **a** distal part of the leaf petiole, **b** median part of the leaf petiole, and **c** proximal part of the leaf petiole. *** (p -value < 0.01)

always with an overlapping distribution of trait measurements (Table 1 and Fig. 4). We did not find significant differences between sexes for the other measured morphological traits, such as area, perimeter, number of indents, and leaf complexity (Table S2, Fig. S3, Fig. S4).

We found significant correlations between the sex and IRD also for all three leaf sections at stage 0 on the independent batch of 40 plantlets (stage 0) sampled at the La Floricola greenhouse (Figure S5 and Table S3).

Discussion

We identified a vegetative morphological trait that reliably discriminates between the sexes of *C. revoluta* at the seedling stage. In a different way from our work, Kaviani et al. (2014) measured only the interval

between leaflets number 29 and 30 of the leaf rachis of adult leaves in off-shoots and found a non-overlapping distribution between the two sexes (Table 1 in Kaviani et al. 2014). In the three plant stages that we examined, we never found any single interval between leaflets that showed a non-overlapping distribution of the IRD (Table S1). However, when we averaged the intervals between leaflets only at the distal part of the leaf petiole (i.e. corresponding to the upper 2–3 intervals at stage 0), we found that, at the juvenile stage 0, the trait has a clear distinction between male and female *C. revoluta* young individuals with no overlapping values between sexes. This suggests that sexual dimorphism in the leaf is already present very early in plant life. However, only a specific section of the leaf (i.e. the distal part) has to be examined for accurate sex determination of *C. revoluta* seedlings. We achieved highly concordant results in two different batches of plants with distinct genetic

Table 1 Results from the t -test or Wilcoxon rank sum test for significant correlation between the sex of individuals and the IRD for plants of stages 1 and 2

Stage	Section of the leaf	df	t	p -value	W
1	Distal part of the leaf petiole	110	7.8486	$2.979e^{-12}$	2360
1	Median part of the leaf petiole	117	7.3342	$3.161e^{-11}$	
1	Proximal part of the leaf petiole			$9.722e^{-13}$	
2	Distal part of the leaf petiole	267.25	2.6672	0.008115	
2	Median part of the leaf petiole	271.68	2.2602	0.0246	
2	Proximal part of the leaf petiole	270.37	2.2263	0.02682	

‘W’ represents the sum of the rankings of values from one of the two groups in the comparison in Wilcoxon rank sum test; ‘t’ represents the ratio of the difference between the means of the two groups in t -test; ‘df’ indicates degrees of freedom of the test

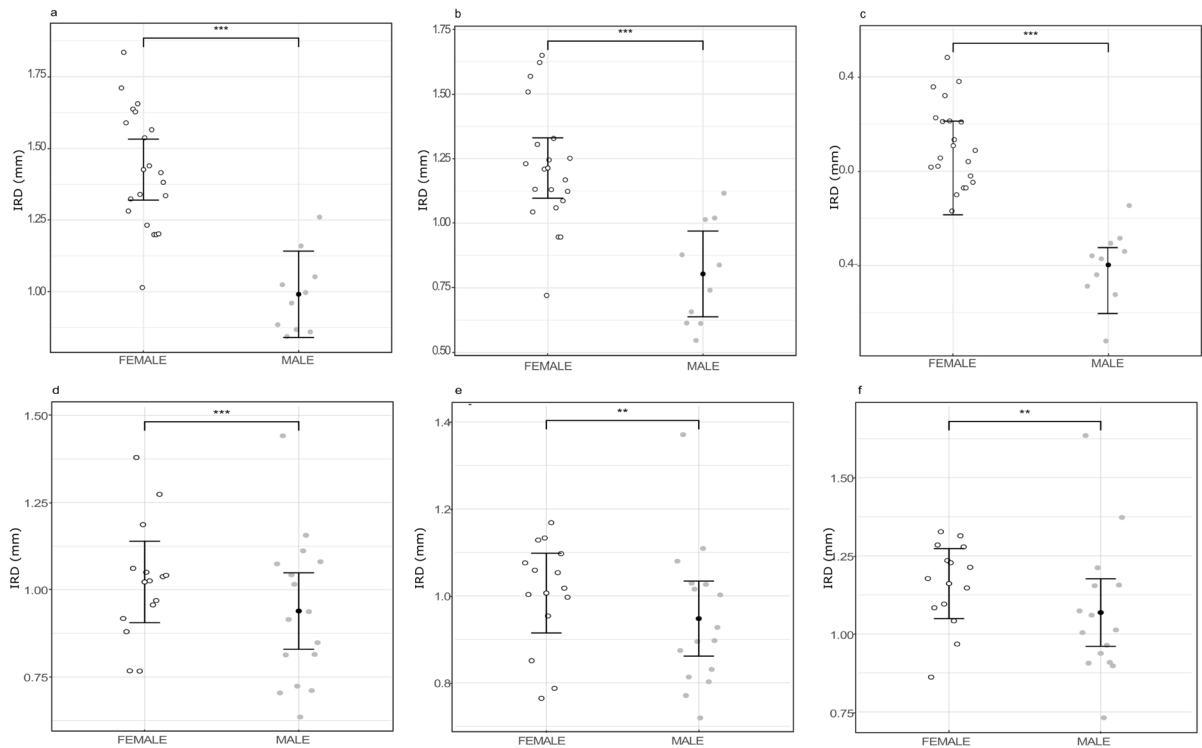


Fig. 4 Dimension of leaf IRD (as the medium of all measurements for each leaf section with error bar, i.e. 95% confidence interval of the mean) of female and male plants for stages 1 (top) and 2 (bottom) in the three leaf sections. **a,d** distal part

of the leaf petiole, **b,e** median part of the leaf petiole, and **c,f** proximal part of the leaf petiole. White dots correspond to female individuals and grey dots represent male individuals. *** (p -value < 0.01), ** (p -value < 0.05)

backgrounds and growth conditions, indicating that this sex-specific trait is not affected by the environment or plant source.

We found that IRD is greater in female individuals but with some exceptions in stage 2 (Table S1). The male leaflets are located almost opposite each other on the central rachis, while the female leaflets are alternate. A complex genetic and molecular network controls the formation of the compound *Cycas* leaf. Cycad leaves resemble fern leaves due to their prolonged apical growth, circinate tips, and leaflets (Stevenson 1990). The marginal position of the leaflets' veins indicates that the leaflets develop exclusively through marginal meristem activity, like in *Ginkgo* (Boyce 2005; Boyce and Knoll 2002). Gene expression of C1KNOX transcription factors (TFs) in *Zamia integrifolia* (Cycadales) was found at the sites of leaflet initiation in older leaf primordia (Bharathan et al. 2002). This may suggest that C1KNOX TFs in Cycads can play a role

in the prolonged apical growth of leaves and the formation of leaflets by establishing new meristems (Romanova et al. 2023). KNOX factors also play a crucial role in the formation of folioles in angiosperms (Hasson et al. 2010). Leaflet formation requires the maintenance of an undifferentiated environment, determined by KNOX factors, and a local accumulation of auxin (Hasson et al. 2010). More precisely, peaks of auxin response contribute to the outgrowth of leaflet primordia, whereas repression of the auxin response is required to inhibit the outgrowth of the regions between leaflets (Barkoulas et al. 2008; Koenig et al. 2009). It could be that plant sex determines a different response to auxin in the early stages of leaf development, contributing to the development of the observed morphological trait dimorphism. It is interesting to note that while the relationship between plant hormones and sex determination is not yet fully understood in gymnosperms, it has been well documented in

flowering plants (i.e. angiosperms) (Song et al. 2013; Yamasaki et al. 2003). Genetic and physiological aspects could therefore explain the presence of sex dimorphism in IRD. Sex dimorphism could evolve as a consequence that males and females have different reproductive roles. Alternatively, a secondary sexual trait could have no direct adaptive significance or could even be counter-adaptive if it is a pleiotropic by-product of physiological or genetic aspects of the mechanism of primary sex determination (Lloyd and Webb 1977). We have no evidence to support an adaptive or non-adaptive significance for this leaf trait. Further studies are also needed to understand why the observed morphological dimorphism is not sustained as the plant grows.

When available, morphological traits as tools for sex differentiation are preferable over molecular identification of sex, particularly in nurseries where thousands of young plants are grown every year. In the next step, the identified phenotypic differences between the sexes could be used to develop a rapid screening tool. For instance, Li and Tang (2017) developed a low-cost system for the three-dimensional (3D) reconstruction of indoor plants and the characterization of their morphological traits such as collar height, length, and leaf area. Alternatively, leaf phenotyping and measurement can be performed directly in the field, using fast and low-cost LiDAR-based systems, such as those introduced by Panjvani et al. (2019) for the measurement of leaf traits (length, width, and area). Both approaches potentially overcome the need to scan the cycad leaves on a flat surface as we did. With our study, we have established the basis for sex identification in *C. revoluta* at an early stage of plant development by employing a sexually dimorphic (measurable) morphological trait. The subsequent step involves creating a faster and more cost-effective identification tool, ideally also in the form of an app for mobile devices based on image recognition and measurement. This functionality can be beneficial not only for setting an efficient and cost-effective system to assist large-scale horticultural companies that produce thousands of plants annually but also for enthusiasts in the field.

Author contribution YDL, DC and AW conceived project and designed experiments; YDL and SC wrote the manuscript; AC performed greenhouse plant measurements; YDL

performed PCR analyses and analyzed data; DC administered the project; all the authors read and approved the manuscript contents.

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Declarations

Conflict of interest The authors declare no competing interests.

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